Isolation and Characterization of the Peptides Derived from the α2 Chain of Chick Bone Collagen after Cyanogen Bromide Cleavage*

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ABSTRACT: The six peptides obtained after cleavage of the α2 chain of chick bone collagen with CNBr have been isolated in approximately equimolar amounts by a combination of ion-exchange and molecular sieve chromatography. The isolated peptides are clearly distinguishable as unique regions of the α2 chain as indicated by chromatographic properties, amino acid composition, and molecular weight. The molecular weights of the peptides range from 319 to 31,000 and total 94,000 in agreement with measured molecular weight of about 95,000 for α chains. All of the amino acids in the α2 chain could be accounted for in the six peptides. The CNBr peptides from the α2 chain of chick bone collagen are clearly homologous to the six CNBr peptides derived from the α2 chain of rat skin collagen.

The peptide from the NH_2-terminal cross-link region, α2-CB1, contains one additional amino acid residue and shows several amino acid substitutions when compared with the homologous peptide from rat skin collagen. In addition, the aldehyde precursor lysyl residue of α2-CB1 from chick bone collagen is partially hydroxylated as was noted for the corresponding residue in the α1 chain of chick bone collagen.

We have previously reported (Miller *et al.*, 1969) the isolation and characterization of ten unique peptides accounting for all of the amino acids and molecular weight of the α1 chain of chick bone collagen after cleavage at methionyl residues with CNBr. As part of a continuing investigation of this collagen whose function is related to the skeletal system the present report details the results of a similar study on the α2 chain.

The nomenclature for designating the peptides derived by CNBr cleavage of collagen is the same as previously used (Butler *et al.*, 1967; Miller *et al.*, 1969). As with the peptides from the α1 chain of chick bone collagen (Miller *et al.*, 1969), the peptides from the α2 chain are numbered on the basis of inferred homology to the six CNBr peptides from the α2 chain of rat skin collagen (Fietzek and Piez, 1969).

Materials and Methods

Source and Preparation of α2. Acid-soluble collagen was obtained from the shafts of the tibiae of 3-week-old lathyritic chicks as previously described (Miller *et al.*, 1967). The α2 chain was isolated by chromatography of 200-mg samples of denatured collagen on CM-cellulose (Whatman, CM32) as described previously (Miller *et al.*, 1969). The column effluent containing the α2 chain was lyophilized, dissolved in 50 ml of 0.1 N acetic acid, and desalted on a 6 × 32 cm column of Bio-Gel P-2 equilibrated with 0.1 N acetic acid.

All chromatography was continuously monitored at 230 mμ by a Beckman DB-G spectrophotometer utilizing a 0.3-ml capacity flow cell and absorbance was recorded on a Beckman 10-in. linear-log potentiometric recorder.

Cleavage with CNBr. The α2 chain was cleaved by incubation at 30° with a 150-fold molar excess of CNBr relative to the methionyl content of the dissolved collagen as previously described (Miller *et al.*, 1969).

Chromatography of CNBr Peptides on Phosphocellulose. The CNBr peptides from the α2 chain were chromatographed on phosphocellulose (Whatman, floc, capacity 7.4 mequiv/g) as previously described (Miller *et al.*, 1969).

Chromatography of CNBr Peptides on CM-cellulose. The more basic CNBr peptides from α2 where eluted as a single peak from phosphocellulose by 0.5 M NaCl (Fietzek and Piez, 1969) and chromatographed on CM-cellulose in 0.2 M sodium citrate buffer (pH 3.6) with a linear gradient of 0.06–0.14 M NaCl as described previously (Miller *et al.*, 1969). The peptide fractions were lyophilized and desalted on Bio-Gel P-2 as described above.

Phosphocellulose Rechromatography of CNBr Peptides Eluted from CM-cellulose. In order to improve the separation of the more basic peptides eluted from CM-cellulose, portions of the CM-cellulose effluent were desalted on Bio-Gel P-2 and rechromatographed on phosphocellulose. The conditions for phosphocellulose chromatography were the same as those described previously (Miller *et al.*, 1969) with the exception that chromatography was performed in 0.06 N (Na^+) acetate buffer (pH 4.8), with a linear

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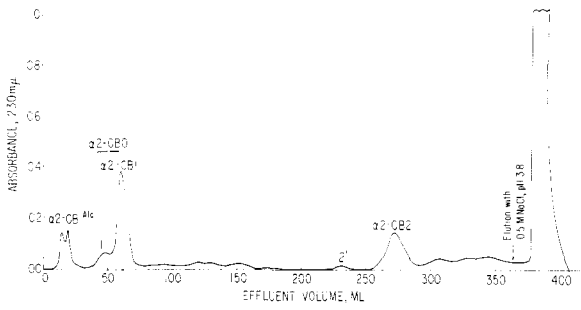

FIGURE 1: Phosphocellulose elution pattern of the CNBr peptides derived from the α2 chain of chick bone collagen. Chromatography was performed in 0.001 M sodium acetate (pH 3.8) using a linear gradient of NaCl from 0.0 to 0.3 M sodium acetate (pH 3.8) using a linear gradient of NaCl from 0.0 to 0.3 M. The total volume of the gradient was 500 ml.

NaCl gradient from 0.0 to 0.1 M. The total gradient volume was 900 ml. The peptide fractions obtained in this manner were lyophilized and desalted on Bio-Gel P-2 as described above.

Molecular Sieve Chromatography on P-4. The smallest of the CNBr peptides from chick bone collagen α2, a tripeptide designated α2-CB0, was most conveniently isolated by chromatography of the lyophilized CNBr digest on 1.7 × 80 cm column of Bio-Gel P-4 as described by Fietzek and Piez (1969).

Molecular Sieve Chromatography on Agarose. The CNBr peptides which had been isolated by ion-exchange chromatography were further purified by molecular sieve chromatography on 1.8 × 230 cm column of agarose beads (Bio-Gel A, 1.5m, 200–400 mesh, Bio-Rad Laboratories). The samples were dissolved in 2.5 ml of 1.0 M $CaCl_2$ (0.05 M Tris, pH 7.5) and were eluted at a flow rate of 15 ml/hr as previously described (Miller *et al.*, 1969). The peptide fractions were lyophilized and desalted on Bio-Gel P-2.

Molecular Weight Determination. The molecular weights of the purified CNBr peptides of α2 were determined by molecular sieve chromatography on an agarose column (see Fietzek and Piez, 1969).

Amino Acid Analysis. Hydrolysates were prepared and analyzed on a single-column amino acid analyzer (see Fietzek and Piez, 1969).

Results

Phosphocellulose Chromatography of CNBr Peptides. Figure 1 illustrates the elution pattern of the CNBr peptides of chick bone collagen α2 obtained by chromatography on phosphocellulose. Four peptides, α2-CB1^{Ald}, α2-CB0, α2-CB1, and α2-CB2, were eluted with the 0.0–0.3 M linear NaCl gradient. The peptides which remained attached to the column at the end of the gradient could be eluted as a single peak by increasing the NaCl concentration at the designated volume to 0.5 M.

The double forepeak contained largely nonprotein material except for trace amounts of α2-CB1^{Ald}, which was isolated as the material appearing near the excluded volume when the forepeak region was desalted on P-2. Peptide α2-CB1^{Ald} was shown to have an amino acid composition identical with that of α2-CB1 (Table I) with the exception of the absence of the partial residues of lysine and hydroxylysine. Peptide α2-CB1^{Ald} therefore presumably represents a small fraction of α2-CB1 in which the lysyl residue has been oxadatively deaminated preparatory to cross-link formation as demonstrated for rat skin collagen (Bornstein and Piez, 1966). The relatively small amount of α2-CB1^{Ald} may be ascribed to the nature of the collagen employed in these studies as it represents a readily solubilized fraction of the bone collagen from lathyritic animals in which cross-link formation is inhibited.

TABLE I: Comparison of the Amino Acid Compositions[a] of α2-CB1^{Ald} and α2-CB1 from the α2 Chain of Chick Bone Collagen.

Amino Acid	α2-CB1^{Ald}	α2-CB1
Aspartic acid	2 (1.8)	2 (1.9)
Serine	1 (0.9)	1 (0.9)
Glutamic acid	1 (1.2)	1 (1.1)
Proline	3 (2.9)	3 (3.0)
Glycine	2 (2.2)	2 (2.2)
Alanine	2 (1.9)	2 (2.0)
Tyrosine	1 (0.7)	1 (0.8)
Phenylalanine	1 (0.9)	1 (1.0)
Hydroxylysine	0	0.5
Lysine	0	0.5
Homoserine	1 (1.1)	1 (1.0)
Total	14	15

[a] Residues per peptide rounded off to the nearest whole number. Actual calculated values are listed in parentheses.

Peptides α2-CB0 and α2-CB1 were not resolved by chromatography on phosphocellulose. As indicated in Figure 1, α2-CB1 chromatographed as two peaks designated α2-CB1′ and α2-CB1. Although the relative heights of α2-CB1′ and α2-CB1 varied in different chromatograms, the total area under the two peaks was constant in relation to the area of α2-CB2. When the phosphocellulose effluent corresponding to α2-CB1′ or α2-CB1 was desalted on P-2, α2-CB0 was eluted from the P-2 column in a volume representing the total fluid volume suggesting that the latter peptide chromatographs between α2-CB1′ and α2-CB1 on phosphocellulose.

Peptide α2-CB2 also exhibited chromatographic heterogeneity on phosphocellulose and was eluted as two peaks designated α2-CB2′ and α2-CB2. α2-CB2′ was always very much smaller than α2-CB2 as illustrated in Figure 1.

Molecular Sieve Chromatography on P-4. Since α2-CB0 could not be obtained free of salt after phosphocellulose chromatography, this peptide was also

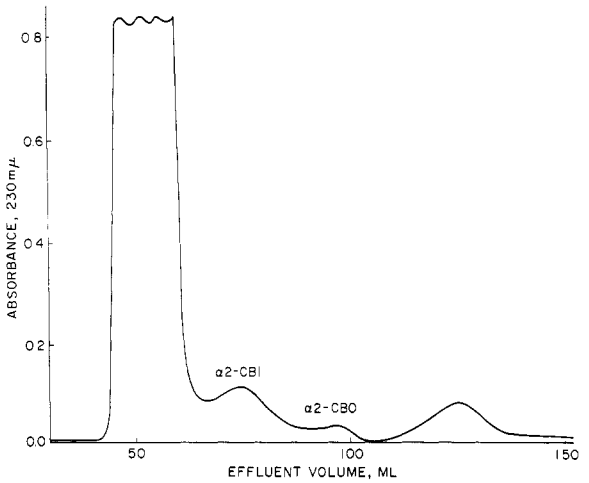

FIGURE 2: Molecular sieve chromatography (Bio-Gel P-4, 180-ml column) of the total CNBr digest of the $\alpha 2$ chain of chick bone collagen. The eluent was 0.1 M acetic acid.

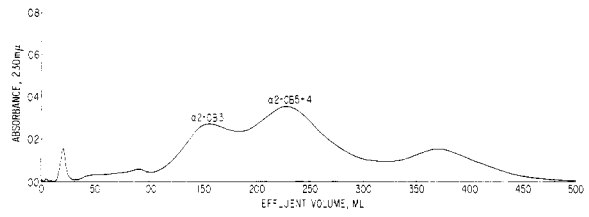

FIGURE 3: CM-cellulose elution pattern of the CNBr peptides derived from the $\alpha 2$ chain of chick bone collagen illustrating the chromatographic properties of the peptides which were not eluted by the gradient from phosphocellulose. Elution was achieved in 0.02 M sodium citrate (pH 3.6) using a linear gradient of NaCl from 0.06 to 0.14 M. The total volume of the gradient was 900 ml.

isolated by chromatography of the total CNBr digest of the $\alpha 2$ chain on Bio-Gel P-4. Figure 2 illustrates the elution pattern. The larger peptides in the mixture were eluted in the void volume of the P-4 column, and $\alpha 2$-CB1 and $\alpha 2$-CB0 were both included in the gel and eluted as the second and third peaks, respectively. Peaks $\alpha 2$-CB0 and $\alpha 2$-CB1 were rechromatographed in the same system to achieve further purification. The peak eluting from P-4 after $\alpha 2$-CB0 was comprised of nonprotein material which was apparently adsorbed to the gel during chromatography.

CM-cellulose Chromatography of the CNBr Peptides Which Were Not Eluted by the Gradient on Phosphocellulose. The three CNBr peptides which were eluted as a single peak from phosphocellulose with 0.5 M NaCl were rechromatographed on CM-cellulose (Figure 3). The first peak contained $\alpha 2$-CB3 while the second larger peak was an unresolved mixture of $\alpha 2$-CB4 and $\alpha 2$-CB5. Amino acid analyses and rechromatography showed heterogeneity with the first part of the peak enriched in $\alpha 2$-CB5 and the latter part enriched in $\alpha 2$-CB4. The third peak was identified as an uncleaved

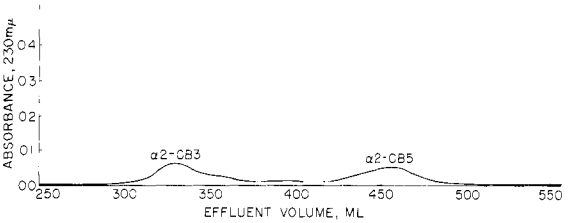

FIGURE 4: Phosphocellulose rechromatography of the 150–200-ml portion of the CM-cellulose effluent (Figure 3). Chromatography was performed in 0.06 N (Na^+) acetate (pH 4.8) with a linear NaCl gradient from 0.0 to 0.1 M. The total volume of the gradient was 900 ml.

peptide by the presence of methonine and a measured molecular weight of approximately 61,000 (see below). Since these peptides were incompletely resolved on CM-cellulose, the column effluent was divided into four fractions for rechromatography on phosphocellulose or agarose.

Phosphocellulose Rechromatography of the CNBr Peptides Eluted from CM-cellulose. Rechromatography of portions of the CM-cellulose effluent on phosphocellulose was performed in 0.06 N (Na^+) acetate buffer (pH 4.8) (see Materials and Methods). The 100–150-ml fraction (Figure 3) contained a single peptide, $\alpha 2$-CB3. Phosphocellulose resolved the 150–200-ml fraction (Figure 3) into two peaks which contained $\alpha 2$-CB3 and a small amount of $\alpha 2$-CB5 (Figure 4). In the latter figure, chromatographic heterogeneity of the eluted peptides can be seen although no significant differences in amino acid composition were noted between various portions of a given peak. This type of heterogeneity of the larger CNBr peptides from the $\alpha 1$ chain of rat skin collagen when chromatographed at pH 4.8 has also been observed (Butler *et al.*, 1967). The mixture of $\alpha 2$-CB4 and $\alpha 2$-CB5 in the 200–250-ml fraction (Figure 3) could not be resolved by rechomatography on phosphocellulose.

Agarose Molecular Sieve Chromatography of the CNBr Peptides Eluted from CM-cellulose. $\alpha 2$-CB4 was isolated by chromatographing the 250–350-ml fraction (Figure 3) on a 1.8 × 230 cm column of agarose beads (Bio-Gel A-1.5m).

The agarose elution pattern is illustrated in Figure 5. The high molecular weight (61,000) peptide eluted in the first peak contained methionine and therefore represents a peptide derived from incomplete CNBr cleavage. The second peak contained $\alpha 2$-CB4 which could be completely resolved from the uncleaved peptide by rechromatography on the agarose column.

Amino Acid Compositions of the CNBr Peptides. Table II contains the amino acid compositions of the six CNBr peptides. Residues per peptide were calculated by using values of amino acids present in low concentration and assuming one residue of homoserine per peptide (with the exception of the COOH-terminal peptide).

$\alpha 2$-CB0 is the smallest peptide, containing one residue each of glycine, leucine, and homoserine. The amino

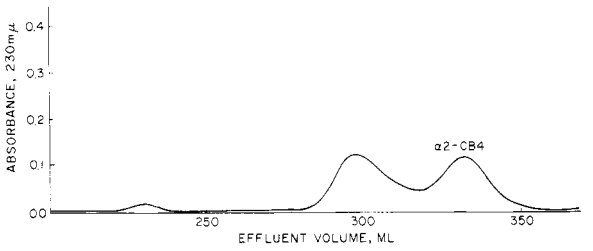

FIGURE 5: Agarose molecular sieve chromatography (Bio-Gel A 1.5m, 585-ml column) of the 250–350 ml portion of the CM-cellulose effluent (Figure 3). The eluent was 1 M $CaCl_2$, pH 7.5 (0.05 M Tris).

acid composition of α2-CB1 indicates that it is homologous to the NH_2-terminal peptide from the α2 chain of rat skin collagen (Bornstein and Piez, 1966; Kang *et al.*, 1967). Variable hydroxylation of the lysyl residue is apparent in α2-CB1 of chick bone collagen as was also noted for α1-CB1 of the bone collagen (Miller *et al.*, 1969). α2-CB2 contains 30 amino acid residues and one-third of these are glycine. Each of the three large peptides contains more than 300 amino acids and one-third of the residues are glycine. Although the larger peptides have similar molecular weights and many similarities in composition, each has distinguishing features which allow it to be differentiated from the other peptides. Peptide α2-CB3 contains less hydroxyproline, serine, leucine, and histidine, and more proline, alanine, valine, and isoleucine than the other large peptides. Peak α2-CB5 is the COOH-terminal peptide as indicated by the absence of homoserine. In addition, α2-CB5 contains the single residue of 3-hydroxyproline from the α2 chain, a single tyrosyl residue and has a relatively high content of histidine.

The last two columns of Table II compare the total number of residues of each amino acid found in the six CNBr peptides from α2 with values obtained from analyses of the whole α2 chain. From this comparison, it is clear that the isolated peptides account, within experimental error, for all of the amino acids in the α2 chain. In addition, since the α2 chain contains five methionyl residues, the six peptides correspond to the expected number.

Molecular Weights of CNBr Peptides. Table III presents the molecular weights of the CNBr peptides as calculated from their amino acid compositions and from their elution volumes on a calibrated agarose molecular sieve column. Agreement of the values obtained by both methods is well within experimental error for each of the peptides. Similarly, the total molecular weight of the isolated peptides is approximately 94,000 by both methods. This value is in good agreement with a reported molecular weight of about

TABLE II: Amino Acid Composition[a] of CNBr Peptides of the α2 Chain of Chick Bone Collagen.

Amino Acid	α2-CB0	α2-CB1	α2-CB2	α2-CB3	α2-CB4	α2-CB5	Total CNBr Peptides	α2[b]
3-Hydroxyproline	0	0	0	0	0	1 (0.5)	1	1 (1.0)
4-Hydroxyproline[c]	0	0	2.2	27	35	35	99	102
Aspartic acid	0	2 (1.9)	3 (2.8)	14	13	16	48	49
Threonine	0	0	0	7 (7.2)	7 (6.9)	7 (6.9)	21	22
Serine	0	1 (0.9)	1 (1.0)	8 (7.8)	11	11	32	32
Glutamic acid	0	1 (1.1)	1 (1.2)	24	21	22	69	73
Proline[c]	0	3.0	2.8	39	32	35	112	116
Glycine	1 (1.2)	2 (2.2)	10	109	102	109	333	338
Alanine	0	2 (2.0)	4 (3.8)	37	33	31	107	110
Valine	0	0	1 (1.0)	11	9 (8.8)	9 (9.2)	30	31
Isoleucine	0	0	0	7 (7.1)	5 (5.1)	6 (5.8)	18	18
Leucine	1 (0.9)	0	1 (0.9)	7 (7.1)	10	12	31	31
Tyrosine	0	1 (0.8)	0	0	0	1 (0.7)	2	2 (2.1)
Phenylalanine	0	1 (1.0)	0	5 (5.0)	4 (4.0)	4 (4.1)	14	14
Hydroxylysine[c]	0	0.5	0.1	2.8	3.2	3.3	10	10
Lysine[c]	0	0.5	0.9	8.9	6.7	5.8	23	23
Histidine	0	0	0	1 (1.0)	2 (2.1)	4 (4.0)	7	7 (7.0)
Arginine	0	0	2 (2.0)	17	17	17	53	53
Homoserine	1 (0.9)	1 (1.0)	1 (1.0)	1 (1.0)	1 (1.0)	0	5	5 (5.1)[d]
Total	3	15	30	326	312	329	1015	1037

[a] Residues per peptide rounded off to the nearest whole number. Actual values are listed in parentheses where less than 10 residues were found. A value of zero indicates less than 0.2 residue. [b] Values in this column have been calculated on the basis of an average residue molecular weight of 91.8 and a molecular weight of 95,000 for the α2 chain. [c] The values for lysine and hydroxylysine and for proline and hydroxyproline are not rounded off where both are present and threre are less than 10 residues since there is evidence for partial hydroxylation giving rise to noninteger values (Butler, 1968; Bornstein, 1967; Miller *et al.*, 1969). [d] Represents methionine in the case of α2.

TABLE III: Molecular Weight of the CNBr Peptides from the α2 Chain of Chick Bone.

Peptide	Amino Acid Anal.	Molecular Sieve Chromatography
α2-CB0	319	319[a]
α2-CB1	1,562	1,600
α2-CB2	2,708	2,900
α2-CB3	29,774	29,000
α2-CB4	28,748	28,000
α2-CB5	30,570	32,000
Total	93,681	93,819

[a] The molecular weight of α2-CB0 was calculated from its amino acid composition only: (Gly, Leu) Met.

95,000 for the whole α2 chain as determined by sedimentation equilibrium (Lewis and Piez, 1964; Kang *et al.*, 1966).

Stoichiometry of Isolated α2 Peptides. In order to estimate the relative yields of the peptides isolated in this study, the amount of homoserine in each of the CNBr peptides from a single α2 preparation was determined. Peptides α2-CB0 and α2-CB1 were isolated from the total CNBr digest by molecular sieve chromatography on P-4 (see Figure 2). The peptides eluted in the void volume of the P-4 column were resolved by chromatography on CM-cellulose into α2-CB2, α2-CB3, and a mixture of α2-CB4 and α2-CB5 (see Figure 3). When α2-CB2 is included in the mixture of peptides for chromatography on CM-cellulose, it is eluted near the front and is well separated from α2-CB3. In Table IV are presented the data on the homoserine content of the peaks isolated in this manner. It is seen that the peptides are present in approximately equivalent amounts. Only 1 equiv of homoserine was found in the peak corresponding to the mixture of α2-CB4 and α2-CB5. However, this is the expected content since α2-CB5 contains no homoserine.

TABLE IV: Stoichiometry of the CNBr Peptides of the α2 Chain of Chick Bone Collagen as Determined by Relative Homoserine Content.

Peptide	Relative Homoserine Content[a]
α2-CB0	0.8
α2-CB1	1.2
α2-CB2	1.0
α2-CB3	0.8
α2-CB4 + α2-CB5	1.0

[a] The data are expressed in terms of the homoserine content of α2-CB2 which was assigned a value of 1.0. α2-CB5 has no homoserine.

Discussion

Cleavage of the α2 chain of chick bone collagen with CNBr gives rise to six unique peptides which have been separated by ion-exchange and molecular sieve chromatography. The peptides have been characterized with regard to amino acid composition and molecular weight and the results indicate that the isolated peptides account for all of the amino acids and molecular weight of the α2 chain. In combination with our previous study of the two α1 chains of chick bone collagen (Miller *et al.*, 1969), the present study completes the isolation and characterization of all the CNBr peptides derived from the collagen molecule of a calcified tissue.

Since the α2 chain of chick bone collagen contains five methionyl residues as compared with nine for the α1 chain, the number of CNBr peptides obtained from the α2 chain is six in contrast to the ten peptides obtained from the α1 chain. Moreover, the methionyl residues of the α2 chain are distributed along the chain in such a manner that three of the six peptides account for approximately 95% of the amino acids and molecular weight of the α2 chain.

We have previously demonstrated (Miller *et al.*, 1969) that the CNBr peptides from the α1 chain of chick bone collagen are homologous to those from the α1 chain of rat skin collagen. The present study allows this comparison to be extended to the α2 chain of the respective collagens as well. Peptides α2-CB1 and α2-CB2 from chick bone are very similar in size and amino acid composition to α2-CB1 and α2-CB2 of rat skin collagen (Bornstein and Piez, 1966). Peptide α2-CB1 from chick bone collagen has one more amino acid residue than the corresponding peptide from rat skin collagen, the lysyl residue is partially hydroxylated and there are several amino acid substitutions. Peptide α2-CB2 from the two collagens have an identical number of amino acid residues but again there are several amino acid substitutions. These peptides from several collagens have been compared in detail (Piez *et al.*, 1968). Of the remaining CNBr peptides from chick bone collagen, the tripeptide, α2-CB0, is identical with α2-CB0 from rat skin collagen (Fietzek and Piez, 1969). It is also clear that α2-CB3, α2-CB4, and α2-CB5 of chick bone collagen are related by amino acid composition and molecular weight to the peptides with the same designation from rat skin collagen (Fietzek and Piez, 1969). For instance, α2-CB3 from both collagens contains a single histidyl residue, α2-CB4 from both collagens contains two histidyl residues, while α2-CB5 from each collagen contains the remainder of the histidyl residues of the α2 chain. In addition, α2-CB5, the COOH-terminal peptide in each collagen, contains a residue of 3-hydroxyproline and, with the exception of α2-CB1, is the only peptide from either collagen which contains tyrosine. Although α2-CB5 from chick bone collagen is clearly homologous to α2-CB5 from rat skin collagen, the bone collagen peptide is eluted before α2-CB4 while the rat skin collagen peptide is eluted after α2-CB4 when the peptides are chromatographed on CM-cellulose. The apparent lower basicity of α2-CB5 from the bone collagen may be related to the number of histidyl residues. The α2 chain of rat skin collagen

contains nine histidyl residues (Fietzek and Piez, 1969) as compared to seven for chick bone α2; α2-CB5 from rat skin collagen contains six histidyl residues while α2-CB5 from chick bone collagen contains four histidyl residues.

In this study it was noted that α2-CB1 and α2-CB2 exhibited chromatographic heterogeneity on phosphocellulose. The most likely explanation for this observation is the existence of an equilibrium between the open and lactone forms of the COOH-terminal homoserine residue in these peptides. At the pH at which chromatography was performed (pH 3.8) one would expect the lactone form to be favored but a small portion would be in the open form.

The order of the CNBr peptides from the α1 chain of chick bone collagen has been tentatively established (Miller *et al.*, 1969) by homology to the rat skin collagen peptides (Piez *et al.*, 1968). With regard to the α2 chain of chick bone collagen, α2-CB1 is probably the NH_2-terminal peptide in the chain since it is homologous to α2-CB1 of rat skin collagen which has been shown to be the NH_2-terminal peptide in that collagen (Bornstein *et al.*, 1966). α2-CB5 is, of course, the COOH-terminal peptide since it lacks homoserine after CNBr cleavage. The order of the intervening four peptides has not as yet been determined.

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